

# Explaining differences in saturation levels for Affymetrix GeneChip<sup>®</sup> arrays

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## ABSTRACT

The experimental spike-in studies of microarray hybridization conducted by Affymetrix demonstrate a nonlinear response of fluorescence intensity signal to target concentration. Several theoretical models have been put forward to explain these data. It was shown that the Langmuir adsorption isotherm recapitulates a general trend of signal response to concentration. However, this model fails to explain some key properties of the observed signal. In particular, according to the simple Langmuir isotherm, all probes should saturate at the same intensity level. However, this effect was not observed in the publicly available Affymetrix spike-in data sets. On the contrary, it was found that the saturation intensities vary greatly and can be predicted based on the probe sequence composition. In our experimental study, we attempt to account for the unexplained variation in the observed probe intensities using customized fluidics scripts. We explore experimentally the effect of the stringent wash, target concentration and hybridization time on the final microarray signal. The washing effect is assessed by scanning chips both prior to and after the stringent wash. Selective labeling of both specific and non-specific targets allows the visualization and investigation of the washing effect for both specific and non-specific signal components. We propose a new qualitative model of the probe-target hybridization mechanism that is in agreement with observed hybridization and washing properties of short oligonucleotide microarrays. This study demonstrates that desorption of incompletely bound targets during the washing cycle

contributes to the observed difference in saturation levels.

## INTRODUCTION

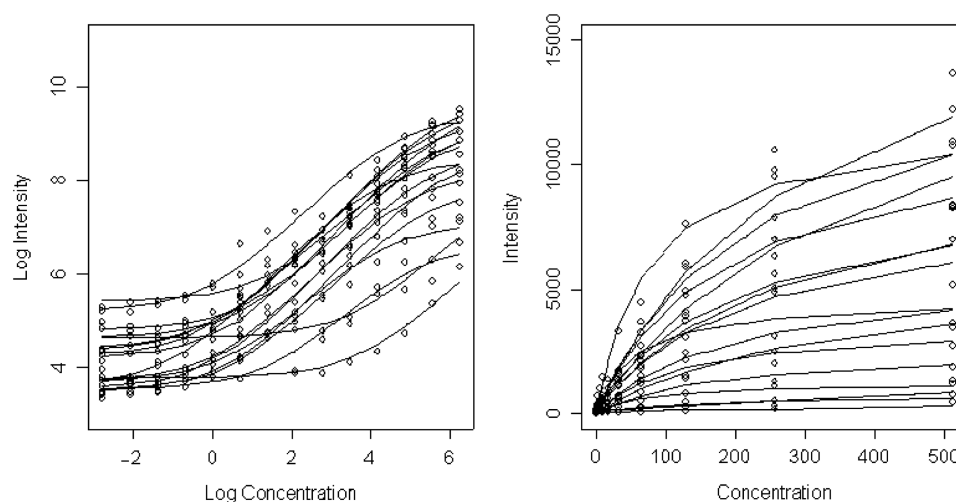
DNA microarrays are versatile tools that allow the massively parallel study of gene expression. Microarray applications rely on the specificity of target-probe binding, when cRNAs form stable duplexes with complimentary surface-attached DNA probes. The availability of the Affymetrix spike-in study (1) has led to a significant effort in exploring the relationship between concentration and microarray signal (2–4). Visual examination of the signal response to concentration in the spike-in data set reveals a nonlinear relationship between transcript abundance and signal; see Figure 1. A simple derivation of the microarray hybridization isotherm is possible by equating the rates of hybridization and denaturation at the surface, and leads to the equation known as the Langmuir adsorption isotherm:

$$[C] = \frac{[O^0] \times K \times [R]}{1 + K \times [R]} \quad 1$$

where  $[C]$  is the number of bound DNA/RNA complexes,  $[O^0]$  is the total number of immobilized oligonucleotides,  $[R]$  is concentration of RNA targets and  $K$  is the equilibrium constant given by Arrhenius' formula  $K = A \times e^{\Delta G/RT}$ ; where  $A$  is a constant pre-factor,  $\Delta G$  is Gibbs' free energy of duplex formation,  $R$  is the Boltzmann gas constant, and  $T$  is absolute temperature. Equation (1) is based on a simple physical 'on/off' model that likely deviates from the complex process of microarray hybridization—herein we refer to this as the simple Langmuir model.

It has been shown that the Langmuir adsorption isotherm captures the shape of GeneChip<sup>®</sup> hybridization (3,5,6). According to the simple Langmuir model, signal response to concentration should saturate at the same

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**Figure 1.** Adsorption isotherms observed for Affymetrix microarrays. The observed adsorption isotherms (signal intensity versus target concentration) for Affymetrix microarrays exhibit different saturation intensities on both the log<sub>2</sub> (left panel) and natural (right panel) scale.

level for all probes. However, as shown in Figure 1, it is apparent that amongst different probes, there is up to a 30-fold difference in saturation levels and variation in these levels was found to correlate with probe sequences (7,8).

Fitting the signal-versus-concentration curves into the Langmuir isotherm equation revealed that the equilibrium constant,  $K$ , does not exhibit strong dependence on the probe sequence as predicted by the simple Langmuir model (5,7). On the contrary, although the nature of the observed saturation range was not addressed experimentally, the saturation levels themselves were successfully predicted by fitting the log<sub>2</sub> saturation intensities to the sum of position-dependent nearest-neighbor stacking energy parameters (4,9). Several hypotheses have been proposed to explain the discrepancies between the simple adsorption model and the observed signal behavior.

Alternative non-Langmuir models claim that saturation intensity is, in fact, constant and discrepancies in predicted parameters arise from fitting the wrong model. For example, Vainrub and Pettitt (10,11) developed a mean field model of the electrostatic effects in oligonucleotide microarrays that describe a non-Langmuir binding isotherm, suggesting that there are substantial differences in hybridization thermodynamics between DNA free in solution and surface-tethered DNA. According to this model, the binding of negatively charged RNA increases the total charge on the microarray surface and significantly affects probe–target-binding kinetics, thus leading to a modified binding isotherm. Another explanation, introduced by Peterson *et al.* (12), is based on the concept of probe–target non-homogeneity, i.e. probes and targets have different lengths, hence binding occurs at varying rates. The Sips model provides a generalization of the Langmuir isotherm in which the single binding energy utilized in the Langmuir version is replaced by a distribution of binding energies. Multi-step reaction models explain the discrepancies between the simple adsorption model and the observed signal behavior by

suggesting that probe–target-binding is a slow, complex multi-step reaction with multiple intermediate states (13–15). This can lead to a dependence shape similar to the Langmuir isotherm, while saturation would then occur at an intensity that is different from the maximum occupancy state (14,12,16).

We hypothesize that the difference in saturation intensity can be explained by the post-hybridization wash. It is generally assumed that the primary purpose of the wash step is to mechanically remove the remains of unbound or loosely bound RNA from the chip surface. We predict that in a typical microarray experiment, a significant portion of non-specific RNA competes with specific RNA for binding sites due to the high complexity of the background, and thus a significant fraction of duplexes are not completely zipped after a standard round of hybridization. Those duplexes are removed during the washing step at a rate dependent on the probe–target duplex binding strength.

The suggested model can be tested by introducing several slight modifications to the existing hybridization protocol. Briefly, the chip is scanned prior to the stringent wash cycle, and scanning is repeated once the wash cycle is complete. If the proposed wash model is correct, we expect to observe a modest intensity variation prior to the wash and a significant sequence-dependent intensity decline following the wash cycle. In this study, we alter the standard Affymetrix fluidics protocol in order to investigate the effect of the wash cycle, target concentration and hybridization time on the final hybridization signal. Scanning the chips prior to the stringent wash cycle allows for quantification of the effect of the wash on both specific and non-specific signal components. By varying the length of the hybridization cycle, the issue of attaining probe–target binding equilibrium can be addressed. Selective labeling of spiked clones and a complex biological background mixture allow the impact of specific and non-specific binding on the total signal to be monitored.

## MATERIALS AND METHODS

In order to elucidate the various factors that influence the kinetics and thermodynamics of target hybridization to surface immobilized probes in a microarray setting, we introduced numerous alterations to the standard Affymetrix protocol for cRNA sample preparation, fragmentation, hybridization, washing, staining and scanning.

To examine the contributions of both the specific and non-specific signal components, we devised an approach to selectively label the targets and complex background while minimizing other differences in cRNA preparation. We exploited the fact that the pOTB7 vector, in which each of the cDNAs employed in this study had been directionally cloned, has a T7 promoter sequence located 3' to the cDNA coding region. This facilitated the use of either Affymetrix's GeneChip® IVT Labeling Kit or Ambion's MEGAscript® T7 Kit to generate antisense cRNAs that differ from one another chiefly in the presence or absence of a biotin label on the transcript.

To study the effects of the wash cycle, we modified the standard fluidics script so as to obtain two measures of hybridization intensities, one prior to and one subsequent to the stringent wash. We further probed the issue of saturation intensity by varying the concentration of the spike-in targets.

### cRNA preparation

Briefly, cDNAs from the *Drosophila* Gene Collection adult testes (AT) library were PCR amplified, pooled and used directly as targets for *in vitro* transcription (IVT). Biotin-labeled and unlabeled antisense cRNAs were generated using either the GeneChip® IVT Labeling Kit or Ambion's MEGAscript® T7 Kit, respectively. Standard protocol, as described in the Affymetrix GeneChip® Expression Analysis manual [17], was followed for IVT, purification and fragmentation of cRNA, with the exception that the volumes of reagents used to generate unlabeled cRNA transcripts were in accordance with Ambion's MEGAscript® protocol. For complex background, total RNA from mouse cerebellum was used as template for cDNA synthesis and IVT resulted in either biotin-labeled or unlabeled cRNA according to the same protocol as described for the cDNA clones. *Drosophila* cRNA transcripts (targets) were then spiked into complex backgrounds at known concentrations prior to fragmentation. See the Supplementary Data for additional details regarding cRNA preparation.

### Hybridization/Washing experiment

According to standard Affymetrix protocol, following the synthesis and purification of labeled cRNAs the product is fragmented and hybridized to the GeneChip® for 16 h, after which the array is subject to a series of washing and staining steps prior to being scanned to obtain hybridization intensities (see Figure 2 for an overview of the protocol).

In particular, there are two types of post-hybridization wash steps: a low stringency wash initiates the wash

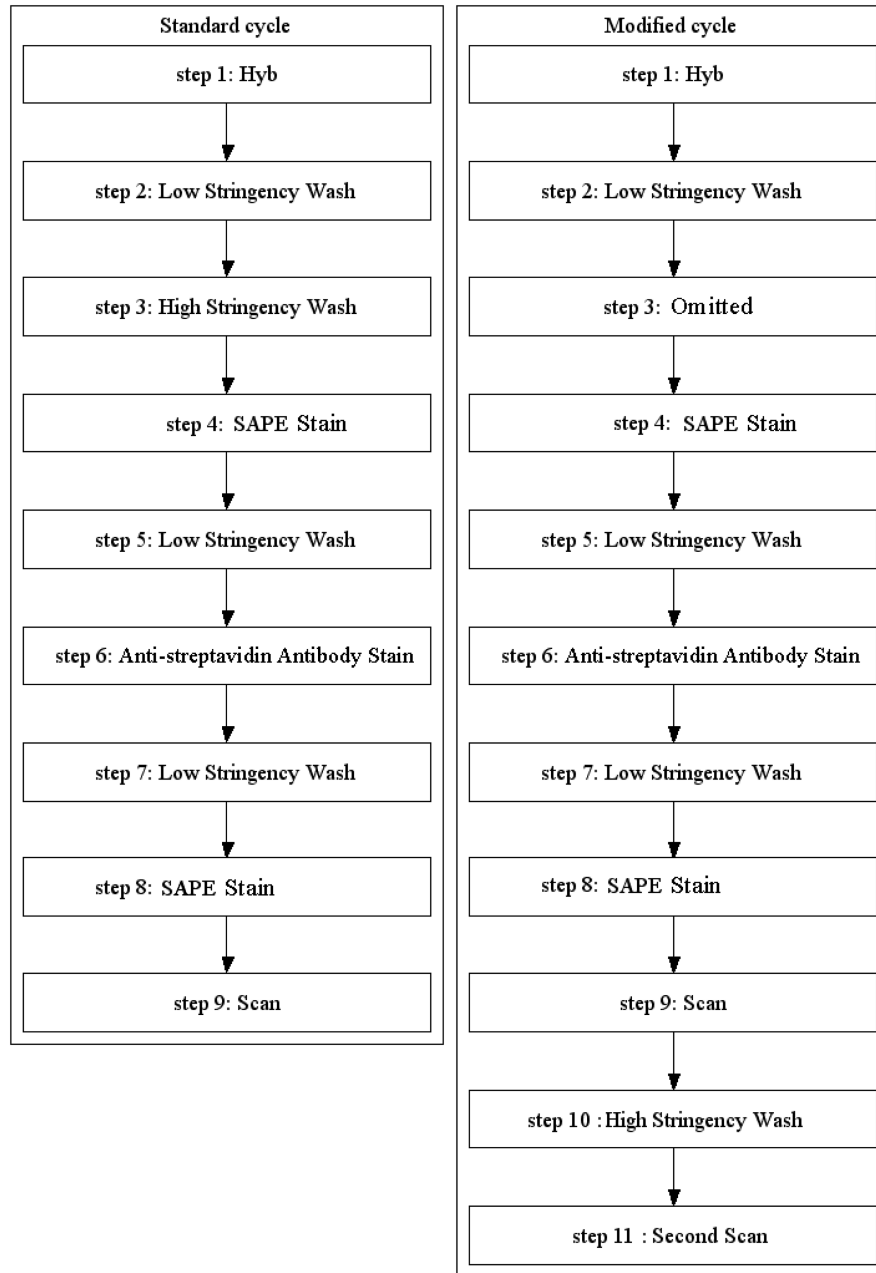
procedure, followed by a high stringency wash in which the salt concentration of the buffer is decreased while the temperature is increased. The GeneChip® is then stained with streptavidin phycoerythrin (SAPE) solution, washed in non-stringent buffer, stained with anti-SAPE antibody, washed again in non-stringent buffer and finally scanned to obtain the hybridization intensities.

We altered the standard wash and hybridization conditions by modifying the fluidics script so that chips are scanned prior to the stringent wash cycle and so that the duration of hybridization is increased in certain experiments. In effect, the modified fluidics script results in omission of the high stringency wash that immediately follows the low stringency wash (Figure 2). All other steps of the wash cycle proceed in the same order as standard protocol dictates with the exception that after the GeneChip® is scanned, the stringent wash is performed and the array is scanned a second time to obtain two measures of hybridization intensities, one prior to and one subsequent to the stringent wash. The concentration of the spike-in targets was also varied in conjunction with the altered wash protocol with target concentrations of either 512 pM, 1024 pM, or 10 nM. When included, complex background was 15 µg or was reduced by a factor of 4. The effect of hybridization time on signal intensity was examined by increasing the duration of hybridization 2.5-fold, from 16 h to 40 h. To prevent evaporation of the hybridization cocktail, Tough Spots™ were placed over the septa on the GeneChip®.

See Table 1 for a description of the concentration of individual clones, the quantity of complex background and the label status of both targets and background in each experiment. Raw CEL data have been deposited in NCBI's Gene Expression Omnibus [18] and are accessible through GEO Series accession number GSE7110, while the processed results are provided as Supplementary Data.

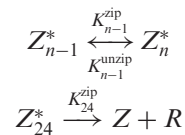
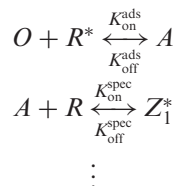
### Model

Due to competitive hybridization and the partial inhibition of probes by non-specific targets during hybridization, a large population of incompletely bound duplexes is expected to occupy the surface of the chip after the hybridization cycle. We suggest a model that explains the observed sequence-dependent nature of saturation intensities by removal of incompletely zipped specific targets during the stringent wash cycle. We hypothesize that hybridization occurs in several stages. The first step involves the non-specific adsorption of RNA fragments to the chip surface. Published results suggest that this step is a relatively fast process with characteristic relaxation times from minutes to a fraction of an hour (19). Due to partial-sequence matches, these non-specific duplexes are stable enough to partially obstruct hybridization sites. We hypothesize that specific hybridization occurs through an intermediate step where both specific and non-specific targets are attached to the probe. Over time, non-specific targets are slowly replaced by specific targets, the rate of replacement depending on the probe sequence. Characteristic relaxation times for such process on the



**Figure 2.** Standard Affymetrix eukaryotic protocol for hybridization, washing, staining and scanning (left) and our modified protocol (right).

order of 10–100 h were previously reported (20,21). Thus, by the end of the standard hybridization cycle a significant fraction of duplexes are not completely zipped and are subsequently removed during the stringent wash cycle. This hybridization model can be described by the following equations:



For each probe, the hybridization process can be described by a chain of chemical equations. Non-specific RNA,  $R^*$ , binds to oligonucleotide probe  $O$  with the rate  $k_{\text{on}}^{\text{ads}}$ , forming complex  $A$ . This complex disassociates to initial components with the rate  $k_{\text{off}}^{\text{ads}}$ . Specific targets,  $R$ , bind to partially occupied probe  $A$  to form an intermediate,  $Z_1^*$ , with rate  $k_{\text{on}}^{\text{spec}}$  and dissociates with rate  $k_{\text{off}}^{\text{spec}}$ . The intermediate complex,  $Z_1^*$ , is transformed



**Table 1.** A summary of key features of the full validation experiment

Experiment	Chip	Clones concentration (labeled/unlabeled)	Number of clones	Foreign background concentration (labeled/unlabeled)	Hybridization time (h)	Scans: before (1) and after (2) stringent wash
Altered protocol benchmark	1	0.5 nM (labeled)	20	15 µg (unlabeled)	16	1/2
	2	0.5 nM (labeled)	20	15 µg (unlabeled)	16	2
Washing cycle experiment	3	0.5 nM (labeled)	20	15 µg (unlabeled)	16	1/2
	4	0.5 nM (labeled)	19	15 µg (unlabeled)	16	1/2
	5	0.5 nM (labeled)	18	15 µg (unlabeled)	16	1/2
Clone concentration experiment	6	1 nM (labeled)	8	0	16	1/2
	7	10 nM (labeled)	4	0	16	1/2
Hybridization time experiment	8	0.5 nM (labeled)	20	15 µg (unlabeled)	16	1/2
	9	0.5 nM (labeled)	20	15 µg (unlabeled)	40	1/2

to fully zipped complex  $Z$  through a series of intermediate states,  $Z_n^*$ , with rate  $K_n^{\text{zip}}$  where the subscript  $n$  corresponds to the number of fully zipped nucleotides for a 25-mer DNA/RNA duplex, neglecting the base pairs terminating the sequence at each end. The hybridization kinetics of the system can be described by a set of differential equations:

$$\begin{aligned}
 \frac{d[O]}{dt} &= -K_{\text{on}}^{\text{ads}}[O][R^*] + K_{\text{off}}^{\text{ads}}[A] \\
 \frac{d[A]}{dt} &= K_{\text{on}}^{\text{ads}}[O][R^*] - K_{\text{off}}^{\text{ads}}[A] - K_{\text{on}}^{\text{spec}}[A][R^*] + K_{\text{off}}^{\text{spec}}[Z_1^*] \\
 \frac{d[Z_1^*]}{dt} &= K_{\text{on}}^{\text{spec}}[A] - K_{\text{off}}^{\text{spec}}[Z_1^*] - K_1^{\text{zip}}[Z_1^*] \\
 &\vdots \\
 \frac{d[Z_n^*]}{dt} &= K_{n-1}^{\text{zip}}[Z_{n-1}^*] - K_n^{\text{unzip}}[Z_n^*] \\
 &\vdots \\
 \frac{d[Z]}{dt} &= K_{24}^{\text{zip}}[Z_{24}^*] \\
 [O^0] &= [O] + [A] + [Z_1^*] + \dots + [Z_n^*] + \dots + [Z_{24}^*] + [Z]
 \end{aligned}
 \tag{2}$$

Here,  $[R]$  and  $[R^*]$  represent the concentration of specific and non-specific RNA fragments respectively, and  $[O^0]$ ,  $[O]$  and  $[C]$  represent the total number of oligos, the number of immobilized oligos and the number of complexes, respectively.

Following hybridization, the stringent wash cycle reduces the number of intermediate products exponentially, similar to the model described in Held *et al.* (7):

$$\begin{aligned}
 A^w &= A \times e^{-k_w^A \times t} \\
 Z_1^{*w} &= Z_1^* \times e^{-k_w^{Z_1^*} \times t} \\
 &\vdots \\
 Z_n^{*w} &= Z_n^* \times e^{-k_w^{Z_n^*} \times t} \\
 &\vdots \\
 Z^w &= Z \times e^{-k_w^Z \times t}
 \end{aligned}
 \tag{3}$$

where  $k_w^{Z_n^*}$  corresponds to the dissociation rate of duplex  $Z_n^*$  and  $t$  is time of the wash cycle. An exact analytical solution of the system of differential equation described above is impractical due to its complexity. We explore

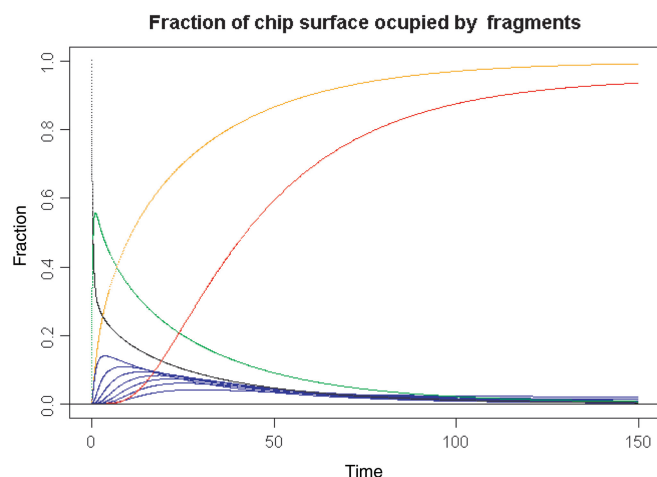
properties of the solution by numerical computation, using matrix methods for systems of equations in R, a language for statistical data analysis (22). Using reaction rate constants similar to those previously reported (19,21,20), we obtained a solution for the fraction of oligos occupied by a particular product as a function of time. Equations of hybridization kinetics depend on the individual properties of each probe so by varying the rates, we can simulate different scenarios of hybridization. For example, the hybridization of mismatch could be simulated by decreasing the forward rate  $K_{13}^{\text{zip}}$  and increasing a backward rate. Alternatively, in order to simulate the behavior of probes with different affinities, one can rescale the time axis.

The described model predicts that for short hybridization times there is a population of incompletely bound duplexes as well as a number of fully zipped duplexes and that over time the number of fully zipped pairs increases. Figure 3 illustrates this model and shows probe occupation with different types of duplexes as a function of time. Initially, non-specific targets (green line) quickly occupy a significant fraction of probes. This is followed by accumulation of specifically bound probe: target duplexes (blue line). The accumulation of fully zipped duplexes (red line) is delayed by the time approximately equal to the sum of relaxation times of all of the intermediate steps. The sum of all intermediate products except for the non-specifically adsorbed targets is shown in orange. Thus, subjecting the chip to a stringent wash cycle is roughly equivalent to shifting from the orange to the red curve; see Figure 3.

## RESULTS

### Altered protocol benchmark

Following sample preparation, as described in the Materials and Methods section, the fragmented target RNA cocktail was hybridized and further processed according to either standard protocol or an altered washing protocol; see Table 1. To assess the effect of signal changes due to the altered protocol, an identical set



**Figure 3.** Numerical solution of system (3). X-axis represents time in hours, Y-axis represents the fraction of chip that is occupied by particular targets. The colored lines represent the following: black, the fraction of unoccupied oligos; green, the fraction of oligos occupied by non-specifically absorbed targets; blue, the fraction occupied by intermediate products (partially zipped complexes with varying degrees of completion); orange, the fraction of all oligos bound to specific targets (fully and partially zipped); red, the fraction occupied by complete fully zipped duplexes.

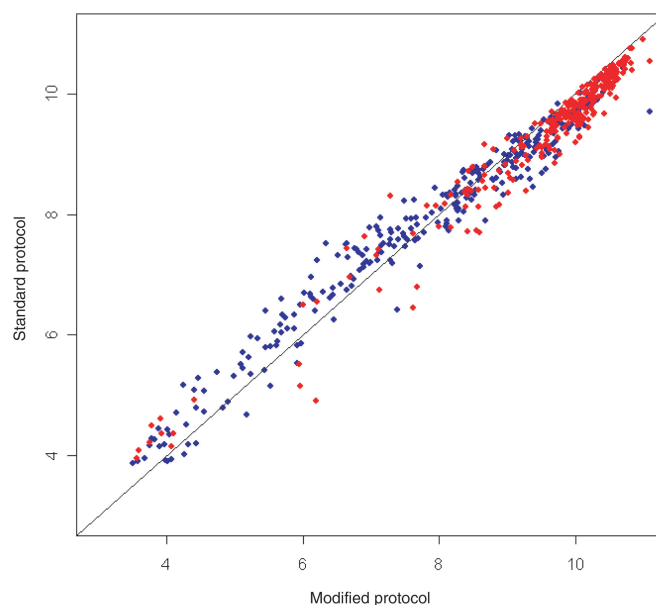
of control mixtures was hybridized using the standard and altered hybridization, washing and staining protocols. The scatter plot of intensities for the same probes on two chips shown in Figure 4 reveals no significant differences between the post-stringent wash signal corresponding to the altered fluidics script and the signal obtained using the standard fluidics script. Thus, these changes in the fluidics script do not introduce any artifacts and do not significantly affect the signal. Hence, we can employ the altered fluidics script to study the effect of the washing cycle on microarray signals.

#### Washing cycle affects both specific and non-specific signal in accordance with probe composition

In order to relate the wash effect (pre/post wash intensity ratio) to the probe sequence composition, a notion of affinity must be introduced. This measure is unknown and could either be measured by direct experimentation or predicted based on probe sequence composition. There are no data available for the measured affinities on the Affymetrix chips, however Zhang *et al.* [4] suggested a method using public data sets to predict them based on sequence composition. According to the simple Langmuir model [Equation (1)] and under the assumption that probes do not attain saturation at 512 pM, we can use the pre-wash intensity as a measure of affinity.

To assess the effect of the washing cycle on the hybridization signal, the experimental conditions were selected so as to mimic the experimental conditions of the Affymetrix spike-in data set.

To assure reproducibility, three independent sets of clones were labeled and spiked into unlabeled complex background; see Table 1 for more details. In particular,

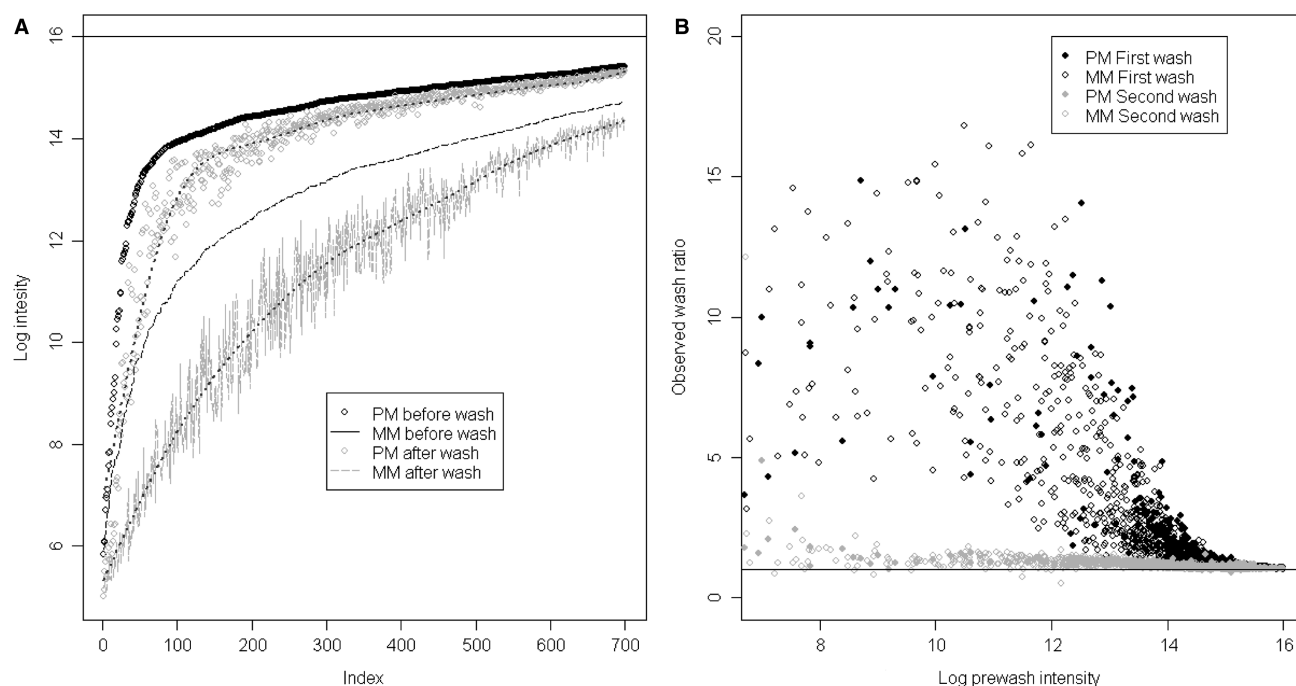


**Figure 4.** Scatterplot of intensities for corresponding probes resulting from the standard and modified protocols. Shown are the  $\log_2$  intensities for spiked clones after the stringent wash from a chip processed according to the standard Affymetrix protocol versus the  $\log_2$  intensities of the same probes on a chip that was processed according to the altered protocol; PM signal [red] and MM signal [blue].

consideration was given to the amount of non-specific background, the concentration of the spike-in clones and the total amount of RNA hybridized. The effect of the stringent wash is shown in Figure 5. Figure 5A shows the  $\log_2$  observed intensities before and after the wash for PM and MM signals. It is clear that the intensities of specific probes signal are altered by the wash cycle and that the post-wash intensity is lower than the corresponding pre-wash intensity.

Figure 5B clearly demonstrates that the wash effect depends on probe affinities and reveals the dependence of the change in intensity resulting from the stringent wash on the pre-wash signal. An extremely pronounced wash effect is observed in which probes with pre-wash intensities ranging from 3000 to 22,000 (on the natural scale) are impacted by a factor of 2–16 as a result of the stringent wash.

In order to further investigate the mechanism underlying the wash process, an additional round of stringent washing and scanning was performed in this experiment to validate the washing model suggested by Held *et al.* (7). In the paper (7), the authors propose that the drop 55 in intensity during the stringent wash step could be described by the exponential decay law. According to the suggested model, the ratio of signal before and after the second wash should then be the same as in the first round of stringent wash. From Figure 5, it is apparent that the second wash does not affect the intensity to the same extent as the first wash; this behavior contradicts theoretical models proposed in the past (7). Surprisingly, MM probes that are only partially bound by design are also not affected by the second wash cycle. This suggests



**Figure 5.** Washing effect. (A) The  $\log_2$  observed intensities for spiked targets in the presence of complex background before the wash for PM (solid black) and MM (dashed black) probes, and after the wash for PM (solid gray) and MM (dashed gray) probes. Intensities are ordered by the pre-wash intensity of PM and MM probes. (B) The observed wash effect (pre/post wash intensity ratio) for spiked probes in the presence of complex background versus the  $\log_2$  observed PM pre-wash intensity. Black and gray dots represent the PM wash effect after the first and second wash, respectively. Open black and gray circles represent the MM wash effect after the first and second wash, respectively.

that the population of probe–target duplexes remaining after the first stringent wash is significantly different from the probe–target duplex population that exists prior to the wash. We believe that those probe–target duplexes that survive the stringent wash are fully bound, whereas prior to the stringent wash the probe–target duplex population contains a significant fraction of partially bound complexes.

#### Comparison of washing effect for non-specific signal

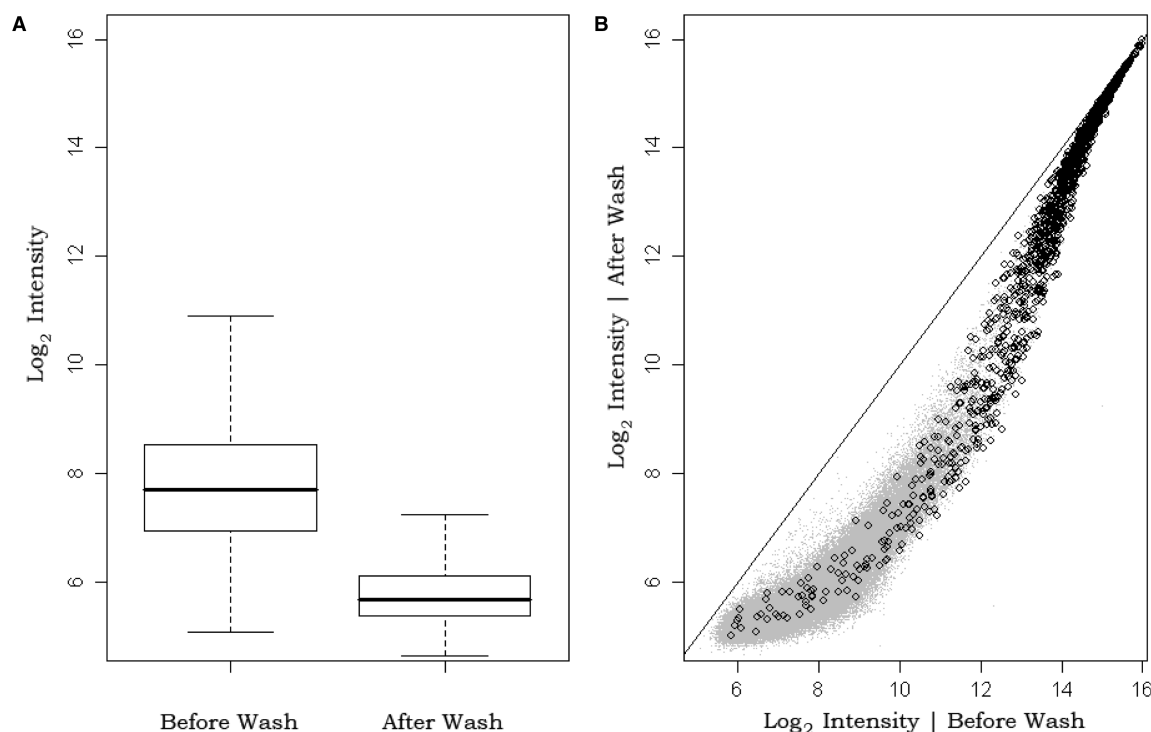
Selective biotin-labeling of spiked clones and non-specific complex background RNA enabled us to explore the properties of specifically and non-specifically formed duplexes. Figure 6 presents a comparison of the signal behavior of specifically and non-specifically bound targets. Figure 6A shows a boxplot of non-specifically bound probe intensities before and after the stringent wash. Prior to wash, the majority of non-specific probes demonstrate a noticeable signal of 7–9 on the  $\log_2$  scale (or 130–500 on natural scale), while after the stringent wash, the interquartile range shifts to the 5–6 range on the  $\log_2$  scale (or 30–60 on natural scale), corresponding to nearly ‘optical background’ level. Figure 6B offers a superimposed scatterplot of specifically and non-specifically bound probe intensities before and after the stringent wash. Examination of this plot reveals that a small fraction of non-specific probes generates a high response, comparable to the intensity of the specific probes, in both pre- and post-wash conditions. Similarly, a fraction of probes representing specifically bound targets demonstrate a low signal, comparable to non-specifically

bound or non-responding probe intensities. We explain these observations by significant cross-hybridization in the first case and presence of non-responding probes and/or probes with no complimentary match in target sequences in the second case. Overlaying before-versus-after wash intensities for specific and non-specific target signals in Figure 6, we observe that the washing properties appear to be uniform across both specifically and non-specifically bound probes. This allows us to conclude that the hybridization/washing mechanism for both specific and non-specific targets is the same and the relationship between pre- and post-wash intensities is universal for all duplexes.

#### Clone concentration and washing effect

According to the simple Langmuir theory, all probes in the pre-wash state are expected to saturate at the same level. However, Figure 5 reveals that probe intensities differ even in the pre-wash state. As mentioned earlier, due to differences in the hybridization kinetics of individual probes, a concentration of 512 pM may not be sufficient to saturate certain probes. To confirm this hypothesis, additional hybridization experiments were performed in which four clones were spiked-in and hybridized to two chips at a concentration of 1 and 10 nM, respectively, each in the absence of complex background; see Table 1.

Figure 7 illustrates the effect of clone concentration on the hybridization signal. Figure 7A compares pre-wash PM and MM  $\log_2$  intensities between chips with 10 and



**Figure 6.** Washing effect for specific and non-specific signal. (A) A boxplot of non-specifically bound probes intensities before and after the stringent wash. (B) before- versus after-wash scatterplot of specifically and non-specifically bound probe intensities; gray dots represent non-specific and black circles represent specific probe intensities.

1 nM clone concentrations. It shows that for high-intensity probes, an increase in concentration does not affect the intensity, i.e. these probes are saturated. However, an increase in concentration results in a nearly proportional increase in the responsiveness of low intensity probes. Thus, the observed difference in pre-wash intensities can primarily be explained by the inability of some probes to saturate at a concentration of 512 pM. The microarray scanner used in our experiments has been well calibrated and the maximum observed intensity was below scanner saturation limits at all times. Figure 7B compares the observed  $\text{log}_2$  difference in wash effects for 10 nM and 1024 pM plotted against the  $\text{log}_2$  pre-wash intensity at 1024 pM. This comparison reveals that the wash effect at the higher concentration is significantly lower than the effect observed at the lower concentration (Wilcoxon signed-rank test,  $P < 0.001$ ) and that PM and MM probes behave similarly in this context.

This observation fits well within the proposed model where the increased concentration of specific clones leads to an increased specific adsorption rate that contributes to increased numbers of fully bound probes. These fully bound duplexes are more stable and less affected by the washing cycle, and hence the washing rates are smaller at higher concentrations.

#### Hybridization time and washing effect

To study the effect of hybridization time on duplex stability, the standard hybridization time (16 h) was extended by a factor of 2.5 (40 h); see Table 1. In order to control for possible experimental variation, the

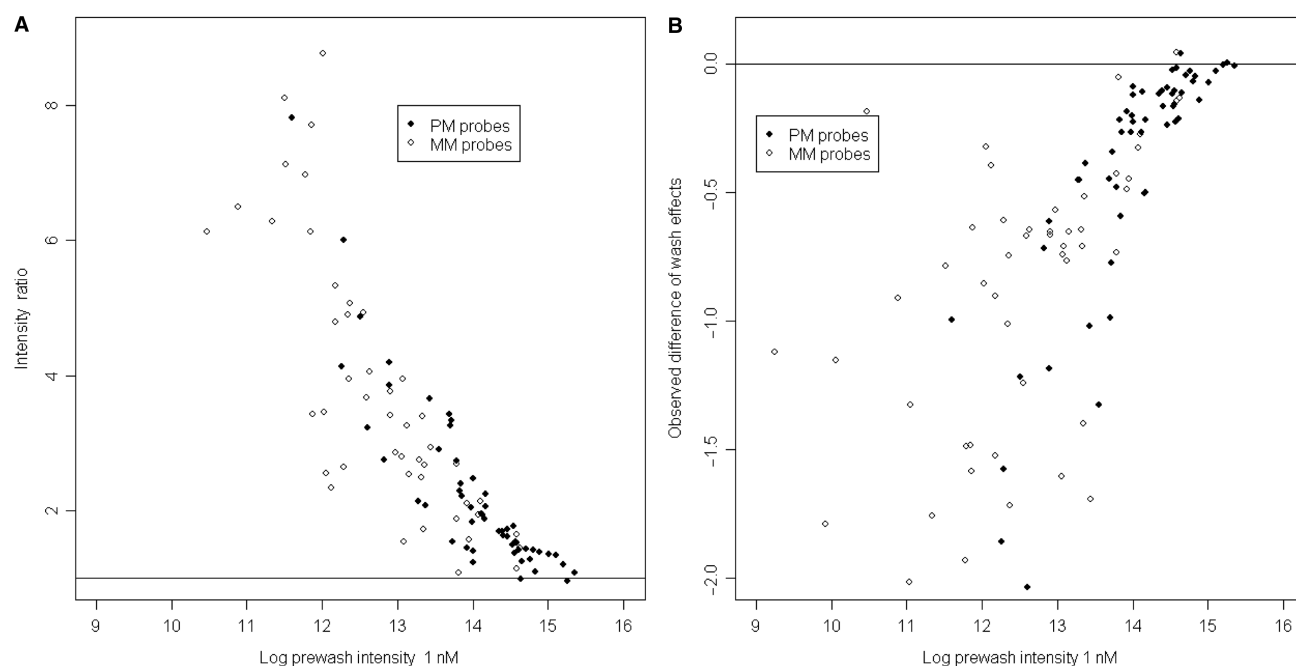
hybridization mixture was prepared as described in the Materials and Methods section, split into two parts and hybridized in parallel on two chips. After 16 h, the first chip was washed, stained and scanned. Following an additional 24 h period, the second chip was washed, stained and scanned.

Examination of Figure 8 reveals that the washing effect was reduced following 40 h of hybridization for probes that were not saturated. Probes that were already near saturation levels were not altered by the wash step, while the overall intensity of the specifically bound probes for 40 h is less than that for 16 h. There is a significant difference observed in washing ratios for 16 h versus 40 h of hybridization (Wilcoxon signed-rank test,  $P < 0.001$ ).

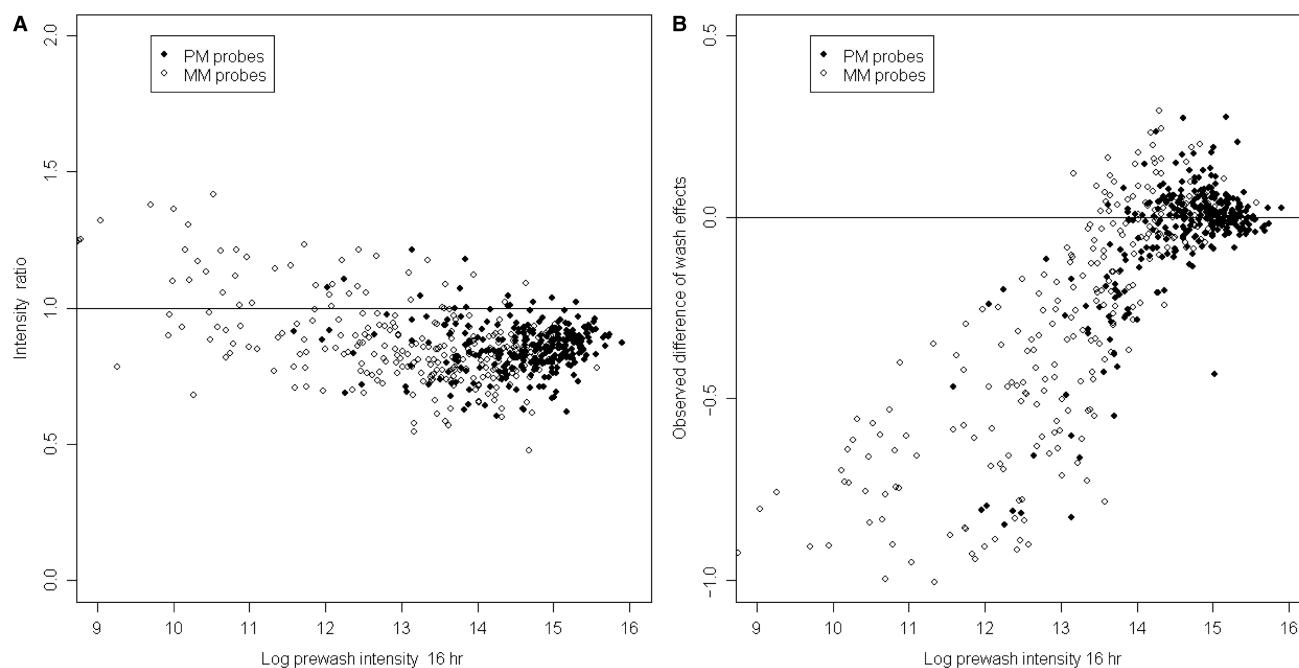
#### DISCUSSION

This study was designed to objectively validate and determine the cause of the variation in saturation levels observed for Affymetrix GeneChip® arrays. Whereas some earlier studies show a quantitative relationship between the saturated intensity signal [4] and the probe sequence composition, experimental validation has not been previously described. Here we study the hybridization and washing steps of the standard Affymetrix protocol to provide a qualitative description of the microarray hybridization mechanism. This is achieved by altering the standard protocols for hybridization, washing and staining. In particular, by modifying the standard protocol we study the effect of the stringent wash, hybridization time, target concentration





**Figure 7.** Concentration effect. (A) The comparison of intensity ratios between hybridization experiments with clone concentrations of 1 nM and 10 nM for PM (black circles) and MM (open circles) probes versus the  $\log_2$  intensity before the wash. (B) The observed  $\log_2$  difference in the wash effect for 1 and 10 nM for PM (black circles) and MM (open circles) probes versus the  $\log_2$  intensity before the wash.



**Figure 8.** Hybridization time effect. (A) The comparison of intensity ratios for spiked probes following 16 and 40 h of hybridization for PM (solid circles) and MM (open circles) probes. (B) shows the  $\log_2$  difference in the wash effect for hybridization experiments with 16 and 40 h hybridization times for PM (solid circles) and MM (open circles) probes.

and the presence of complex background on the microarray hybridization signal. We establish that the major factor contributing to the discrepancy between the signal predicted by the simple Langmuir model and the intensity observed in public Affymetrix spike-in

experiments is the stringent wash cycle, which results in decreased signal in accordance with probe affinity. By increasing the duration of hybridization we observe that low-affinity probes do not reach full equilibrium over the standard 16 h cycle and this partially explains the

variation in saturation levels prior to the stringent wash step. By increasing the clone concentration we gain insight into the mechanism of probe–target binding kinetics. We observe a diminished washing effect at higher spike-in concentrations, an effect that cannot be dismissed as the result of normal variability.

Consecutive stringent wash experiments, in which chips are subjected to two rounds of stringent washing and scanning, demonstrate the heterogeneous nature of the formed duplexes. We observe that 60–95% of specifically bound targets are washed off during the first stringent washing cycle, while a second stringent washing cycle only mildly alters the signal. In light of this observation, we can hypothesize why the observed energy of duplex formation for microarrays appears to be smaller than the one observed in solution: only a limited fraction of duplexes become fully zipped during hybridization. This would also explain the discrepancies between theoretically calculated equilibrium constants and those observed in experimental data.

Our findings explain the mysterious difference in saturation intensities and provide insight into the theoretical and statistical modeling of Affymetrix microarray hybridization signals. Investigators should consider the nature of the hybridization mechanism, the heterogeneity of probe–target interactions, and the effect of the stringent wash when modeling hybridization signals.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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